Antimicrobial activity of saquayamycins produced by *Streptomyces* spp. PAL114 isolated from a Saharan soil

Activité antimicrobienne des saquayamycines produites par Streptomyces spp. *PAL114 isolé d'un sol saharien*

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KEYWORDS
Streptomyces;
Saquayamycins;
Pathogenic microorganisms;
Antimicrobial activity;
Antifungal activity

Summary A new strain of actinomycete designated PAL114, producing antimicrobial compounds, was isolated from a Saharan soil in Ghardaïa, Algeria. Morphological and chemical studies showed that this strain belonged to the genus *Streptomyces*. Two bioactive compounds, named P41A and P41B, were extracted by dichloromethane from the cell-free supernatant broth of strain PAL114 and were purified by HPLC. Minimum inhibitory concentrations of the pure antibiotics were determined against yeasts, filamentous fungi and bacteria, most of which are pathogenic or toxigenic for human and multiresistant to antibiotics. The strongest activities were observed against *Candida albicans* M3 and *Bacillus subtilis* ATCC 6633. The chemical structures of the compounds were determined by spectroscopic analysis of UV—visible and ^{1}H and ^{13}C NMR spectra and spectrometric analysis of mass spectrum. The compounds P41A and P41B were identified as saquayamycins A and C, respectively. These compounds belong to the aquayamycin-group antibiotics, which are known in the literature for their anticancer and antibacterial activities.

MOTS CLÉS
Streptomyces ;
Saquayamycines ;
Microorganismes

Résumé Une nouvelle souche d'actinomycète désignée PAL114, produisant des composés antimicrobiens, a été isolée à partir du sol saharien de Ghardaïa, Algérie. Des études morphologiques et chimiques ont permis de rattacher cette souche au genre *Streptomyces*. Deux composés actifs, nommés P41A et P41B, ont été extraits par le dichlorométhane à partir du filtrat de culture de la souche PAL114 et purifiés par HPLC. Les concentrations minimales inhibitrices

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des fractions actives pures ont été déterminées contre des levures, des champignons filamenteux et des bactéries, dont la plupart sont pathogènes ou toxinogènes pour l'homme et multirésistants aux antibiotiques. L'activité la plus importante a été observée contre *Candida albicans* M3 et *Bacillus subtilis* ATCC 6633. Les structures chimiques des molécules ont été déterminées par analyses spectroscopiques (UV—visible et spectres RMN du ^{1}H et du ^{13}C) et analyse spectrométrique (spectre de masse). Les composés P41A et P41B ont été identifiés comme étant les saquayamycines A et C, respectivement. Ces composés appartiennent aux antibiotiques du groupe des aquayamycines, connus dans la littérature pour leur activité anticancéreuse et antibactérienne.

Introduction

Considering the increasing resistance of pathogens to antibiotics, research has intensified in order to discover new bioactive molecules. Among these pathogenic microorganisms, fungi and yeasts cause serious diseases that affect humans. Mycoses have increased dramatically during the last decade. They rank fourth among nosocomial infections [23]. Bacterial infections pose also clinical problems, since many works in the world and in Algeria have reported the emergence of new bacterial strains resistant to multiple antibiotics, including some cephalosporins of third and fourth generation [12,16,19].

Because of still perceptible deficiencies, which are observed during the treatment of fungal and bacterial diseases, and considering the resistance of microorganisms to antibiotics, the current works are oriented toward the search for new bioactive molecules. Among the molecules with antimicrobial activity, the range of the antifungal compounds is much more limited than those of the antibacterial compounds [6]. This is why new non-toxic antifungal molecules should be sought.

Actinomycetes are Gram-positive bacteria with a percentage of guanine—cytosine higher than 55%, and most of them produce mycelia. They are particularly interesting due to their capacity to produce secondary metabolites with diverse chemical structures [25]. They are especially known for the production of antibacterial and antifungal antibiotics. It has been estimated that about two-third of the natural antibiotics have been isolated from actinomycetes [22]. The genus *Streptomyces* is known as the producer of the largest number of antibiotics. It produces about 80% of the antibiotics secreted by actinomycetes [7].

Our previous work showed the richness of antibiotic-producing actinomycetes in the Algerian Saharan soil [18]. The present work aims to study the antifungal and antibacterial activities of a new strain of actinomycete against several pathogenic and toxigenic microorganisms to human, and to determine the chemical structure of the bioactive molecules produced by this strain.

Materials and methods

Isolation and features of the actinomycete strain

A new strain of actinomycete, designated PAL114, was isolated from a Saharan soil collected from Béni-isguen (latitude, 32°27′N; longitude, 03°40′E; altitude, 468 m), Ghardaïa province (South of Algeria). The dry soil sample was suspended in sterile deionized water and diluted. Aliquots (0.2 mL) of each dilution were spread onto chitin—vitamin agar [8]. The medium was supplemented with cycloheximide (80 mg/L) to inhibit the growth of any fungi. The plates were incubated at 30 °C for two weeks.

The strain PAL114 was identified at the genus level on the basis of morphological and chemical features. Both of these criteria are widely used to identify the genera of actinomycetes [11].

The morphological and cultural characteristics were observed by naked-eye examination of 14 day-old cultures grown on various International *Streptomyces* Project (ISP) media: yeast extract—malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salts—starch agar (ISP-4) and glycerol—asparagine agar (ISP-5) [21], and also on Bennett medium [26]. The micromorphology was observed by light microscopy.

For chemotaxonomic analyses, biomass was obtained by growing strain PAL114 in ISP-2 broth in shake flasks [21] and incubated at 30 °C for 4 days. Diaminopimelic acid isomers and whole-cell sugar pattern were analyzed according to the methods of Becker et al. [3] and Lechevalier and Lechevalier [13], respectively.

Target microorganisms

Except strains with ATCC numbers, all other target microorganisms were isolated from diseased patients undergoing antibiotic therapy, from medical equipment and from the hospital environment sampled in five Algerian hospitals located in Algiers and Béjaïa. The used target microorganisms were yeasts (*Candida albicans* M1, M2, M3, IPA200 and *Saccharomyces cerevisiae* ATCC 4226), filamentous fungi (*Aspergillus carbonarius* M333, *Aspergillus flavus* AF3, *Fusarium culmorum* FC200 and *Penicillium glabrum* PG1) and bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* S1 and *Klebsiella pneumoniae* E40). These microorganisms were mostly pathogenic or toxigenic for humans and multiresistant to antibiotics. All strains of *C. albicans* are resistant to cycloheximide, itraconazole, nystatin, terbinafine and thioconazole, and sensitive to amphotericin B. *Saccharomyces cerevisiae* ATCC 4226 resists to the same antifungal compounds, except cycloheximide and amphotericin B. *Aspergillus carbonarius* M333, *A. flavus* AF3 and *Penicillium glabrum* PG1 resist to cycloheximide but *Fusarium culmorum* FC200 resists to cycloheximide, itraconazole, nystatin, terbinafine and amphotericin B. *Bacillus*

subtilis ATCC 6633 is a sensitive bacterium and resists only to neomycin. *Staphylococcus aureus* S1 resists to gentamicin, kanamycin, neomycin, spiramycin and vancomycin. *Klebsiella pneumoniae* E40 resists to cefotaxime and ceftazidime.

Production and purification of bioactive compounds

The production of bioactive compounds was conducted in ISP-2 broth. This medium was chosen among several others because of its very good results in our preliminary work. A seed culture was prepared with the same medium and used to inoculate 20 Erlenmeyer flasks of 500 mL, each containing 100 mL of medium. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C. The extraction of the active compounds was carried out on the fifth day (previously determined as being the day of the optimal production) by centrifugation (5000 *g*, 20 min) of the culture broth to eliminate cells. The cell-free supernatant was extracted with an equal volume of dichloromethane. The organic extract was concentrated to dryness by a rotary evaporator under a vacuum at a temperature lower than 40 °C. The resulting dry extract was recuperated in 0.5 mL of methanol and bioassayed against *Candida albicans* M3, *Aspergillus carbonarius* M333 and *Bacillus subtilis* ATCC 6633 by paper disk diffusion method (disk diameter, 6 mm) at the rate of 30 μL per disk. These three target microorganisms were selected from the twelve previously cited because they represent the three groups of microorganisms studied (bacteria, yeast and filamentous fungi), and also because of their higher sensitivity to the antibiotic activity (noticed in preliminary work), thus, facilitating the detection of bioactive compounds.

Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60HF 254—366; 20 × 20 cm) was employed for the partial purification of antimicrobial products. TLC plates were developed in the solvent system ethyl acetate—methanol (100:15 v/v). The developed TLC plates were air dried overnight to remove all traces of the solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence), and the bioactive spot was detected by bioautography [5]. The retention factor (R_f) of the bioactive spot was measured.

The final purification of bioactive compounds was carried out by Waters reverse phase HPLC using an XBridge C18 (5 μm) column (200 × 10 mm, WATERS) with a continuous linear gradient solvent system from 20 to 100% methanol in water, a flow rate of 2 mL/min and UV detection at 220 nm and 254 nm. All the peaks fraction were collected and tested against *Candida albicans* M3, *Aspergillus carbonarius* M333 and *Bacillus subtilis* ATCC 6633, in order to detect the active fractions and distinguish them from the non-active fractions. The choice of these three target microorganisms was already justified (as described above).

Structure determination of the active compounds

These analyses were carried out with the antimicrobial compounds purified by HPLC. The UV spectrum was given with a SHIMADZU UV1605 spectrophotometer. The mass spectrum was recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) with nanospray ion electro-spray ionization (ESI) source (positive and negative ion mode). ^{1}H and ^{13}C NMR spectroscopy were used for the characterization of the active molecules. NMR sample was prepared by dissolving 3 mg of each purified compound in 600 μL of CD_3OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ^{1}H, ^{31}P, BB). All the chemical shifts for ^{1}H and ^{13}C are relative to TMS using ^{1}H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ^{1}H and ^{13}C signals were assigned on the basis of chemical shifts, spin-spin coupling constants, splitting patterns and signal intensities, and by using $^{1}H—^{1}H$ COSY45, $^{1}H—^{13}C$ HSQC and $^{1}H—^{13}C$ HMBC experiments. Gradient-enhanced ^{1}H COSY45 was realized included 36 scans per increment. $^{1}H—^{13}C$ correlation spectra using a gradient-enhanced HSQC sequence (delay was optimised for $^{1}J_{CH}$ of 145 Hz) was obtained with 200 scans per increment. Gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (340 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of pure bioactive compounds were performed using conventional agar dilution method [17] on the twelve target microorganisms cited above (see paragraph about target microorganisms). These latter were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for yeasts and filamentous fungi, containing different concentrations of active compounds (1, 2, 5, 10, 20, 30, 50, 75 and 100 μg/mL). After a growth period of 24—48 h at 37 °C for bacteria and 48—72 h at 28 °C for fungi, the plates were examined for growth and the lowest antibiotic concentration that inhibited the growth of each organism was determined. Mueller Hinton and Sabouraud media without active compounds and inoculated with target microorganisms was used as a control treatment.

Results

Identification of actinomycete to the genus level

The actinomycete strain PAL114 formed a well-developed and ramified aerial mycelium with long spiraled chains of spores carried by sporophores. The spores were oval and 1—1.5 × 0.6—1 μm in size. The sporulation was good on all the media used. The substrate mycelium was non-fragmented. The strain showed good growth on ISP-2, ISP-3, ISP-4, ISP-5 and Bennett media. The aerial and substrate mycelia were light to medium gray and light brown, respectively. Diffusible pigment with light brown color was produced on ISP-2, ISP-3 and Bennett media. The chemotaxonomic study showed the presence of LL-diaminopimelic acid isomer and glycin in the cell wall and the presence of galactose, glucose and ribose in the whole-cell hydrolysates.

Detection and purification of bioactive compounds

The dichloromethane extract of the culture filtrate, previously dried and then redissolved in 0.5 mL of methanol, was inoculated on paper disks (6 mm diameter) at a rate of 30 μL per disk. The diameters of inhibition obtained were 20 mm for *Bacillus subtilis* ATCC 6633, 20 mm for *Candida albicans* M3 and 12 mm for *Aspergillus carbonarius* M333 (disk diameter included).

On silica gel thin-layer chromatogram, the dichloromethane extract migrated and gave one bioautographic fraction, which was active against *Candida albicans* M3, *Aspergillus carbonarius* M333 and *Bacillus subtilis* ATCC 6633. This fraction (R_f = 0.9 in ethyl acetate—methanol, 100:15 v/v) was injected in HPLC apparatus. Two active compounds against these three microorganisms, named P41A and P41B, were detected and eluted with 80% of methanol in water at a retention times of 41.05 min and 41.56 min, respectively. A quantity of 3 mg of each compound was obtained from 2 L of the culture filtrate.

Structure determination of bioactive compounds

The UV—visible spectra in methanol (data not shown) exhibited a maximal absorption at 218, 317 and 425 nm for P41A and at 218, 316 and 425 nm for P41B. The ESI—MS spectra contained an ion peak at *m/z* 819.1 $[M-H]^-$ for P41A and at *m/z* 823.2 $[M-H]^-$ for P41B (Fig. 1). Thus, the molecular weights of these two compounds were M = 820 for P41A and M = 824 for P41B.

The ^1H and ^{13}C chemical shifts of P41A and P41B compounds are given in Table 1 and their structures can be seen in Figs. 2 and 3.

The HSQC and HMBC spectra show 36 carbon signals for P41A and P41B molecules. It was possible to discern 4 ketone groups (δ_c 182.4 to 205.2), 3 hydroxyl groups (δ_c 70.9 to 157.8), 7 ether functions (δ_c 66.7 to 98.9) 4 sp^2—hybridized carbons (δ_c from 116.4 to 145.2) and 11 sp^3—hybridized carbons (δ_c 13ś9 to 42.6) for P41a molecule, 5 ketone groups (δ_c 182.4 to 205.2), 7 ether functions (δ_c 66.7 to 95.0) and 2 hydroxyl groups (δ_c 70.9 to 157.8) 6 sp^2—hybridized carbons (δ_c from 116.4 to 145.2) and 9 sp^3—hybridized carbons (δ_c 13ś9 to 42.6) for P41b compound. The hydrogens of the hydroxyl group are not observed due to the rapid exchange with MeOD. The 2D $^1H—^1H$ and $^1H—^{13}C$ (see Figs. 2 and 3) permitted to establish the connectivity between all the groups of the P41A and P41B molecules.

Minimum inhibitory concentrations

Minimum inhibitory concentrations of antimicrobial compounds purified by HPLC are summarized in Table 2. The MIC values obtained against the target microorganisms the same for both active compounds. They were comprised between 30—50 μg/mL for yeasts, 75—100 μg/mL for filamentous fungi and 30—50 μg/mL for Gram-positive bacteria. Gram-negative bacterium tested was resistant. The

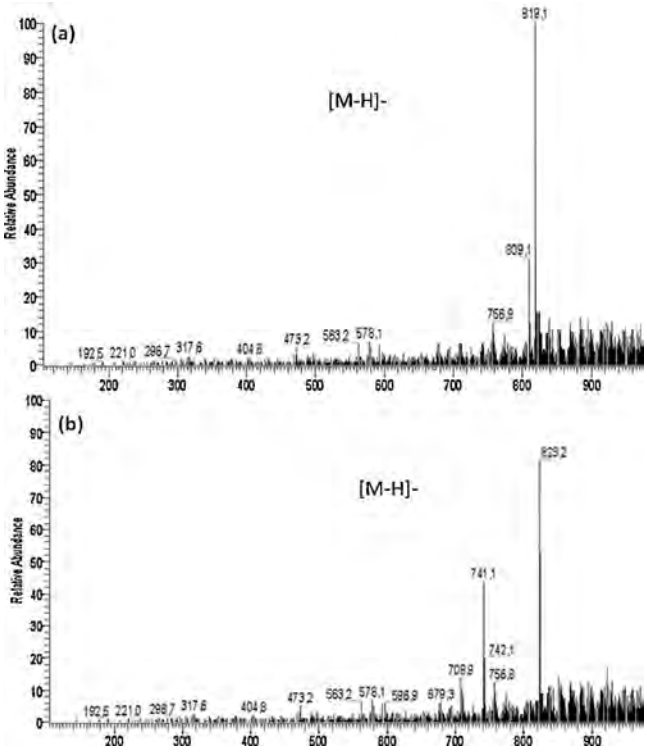

Figure 1 Mass spectrometry profile of P41A (a) and P41B (b) compounds produced by the actinomycete strain PAL114.
Spectre de masse des composés P41A et P41B produits par la souche d'actinomycète PAL114.

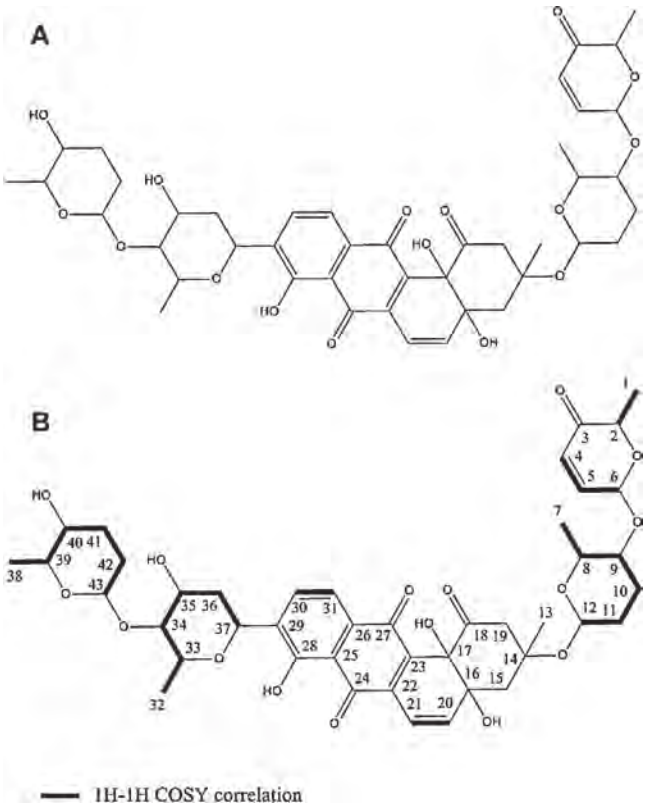

Figure 2 Structure of antibiotic P41A (A) and COSY correlations (B).
Structure de l'antibiotique P41A (A) et corrélations COSY (B).

Table 1 ^{1}H and ^{13}C NMR data assignments of P41A and P41B compounds in CD_3OD at 298 K. See Figs. 2 and 3 for numbering of hydrogen and carbon atoms.
Données RMN ^{1}H and ^{13}C des composés P41A et P41B dissous dans du CD_3OD à 298 K.

^{1}H and ^{13}C number	^{1}H chemical shift (ppm)		^{13}C chemical shift (ppm)	
	P41A	P41B	P41A	P41B
1	1.33	1.33	13.91	13.91
2	4.61	4.61	70.10	70.10
3	—	—	197.27	197.27
4	7.03	7.03	141.1	141.1
5	6.08	6.08	126.1	126.1
6	5.35	5.35	95.0	95.0
7	1.25	1.25	15.9	15.9
8	4.24	4.24	66.7	66.7
9	3.71	3.71	76.4	76.4
10	1.50	1.50	24.0	24.0
11	1.98	1.98	24.0	24.0
12	5.25	5.25	91.8	91.8
13	2.39	2.39	24.8	24.8
14	—	—	81.9	81.9
15	2.00—2.32	2.00—2.32	42.61	42.61
16	—	—	79.8	79.8
17	—	—	79.5	79.5
18	—	—	205.2	205.2
19	2.73—2.93	2.73—2.93	50.1	50.1
20	6.91	6.91	116.4	116.4
21	6.41	6.41	145.2	145.2
22	—	—	139.3	139.3
23	—	—	138.5	138.5
24	—	—	182.4	182.4
25	—	—	137.7	137.7
26	—	—	131.0	131.0
27	—	—	182.4	182.4
28	—	—	157.8	157.8
29	—	—	114.0	114.0
30	7.89	7.89	132.8	132.8
31	7.62	7.62	118.6	118.6
32	1.38	1.38	17.4	17.4
33	3.57	3.57	74.9	74.9
34	3.14	3.14	86.8	86.8
35	3.83	3.83	71.0	71.0
36	1.41—2.49	1.41—2.49	38.9	38.9
37	4.95	4.95	71.0	71.0
38	1.26	1.33	16.7	13.9
39	3.92	4.61	70.5	70.1
40	3.24	—	70.9	197.3
41	1.84	7.03	26.7	144.1
42	1.31—1.94	6.08	29.4	126.1
43	5.02	5.35	98.9	95.0

most sensitive microorganisms were *Bacillus subtilis* ATCC 6633 (30 μg/mL) and *Candida albicans* M3 (30 μg/mL).

Discussion

The strain PAL114 has an aerial mycelium producing chains of spores, each carried by sporophores, and non-fragmented substrate mycelium. This strain possesses in its wall, the LL-diaminopimelic acid isomer and glycin, but does not have in its cells diagnostic sugars, such as arabinose—galactose, xylose—arabinose and rhamnose—galactose couples, or madurose. These results indicate that strain PAL114 has a chemotype IC as defined by Lechevalier and Lechevalier [13].

Based on the morphological and chemical characteristics, strain PAL114 belonged to the genus *Streptomyces* [9].

Species of the genus *Streptomyces* are mainly known for the production of bioactive molecules [22]. The antibiotics

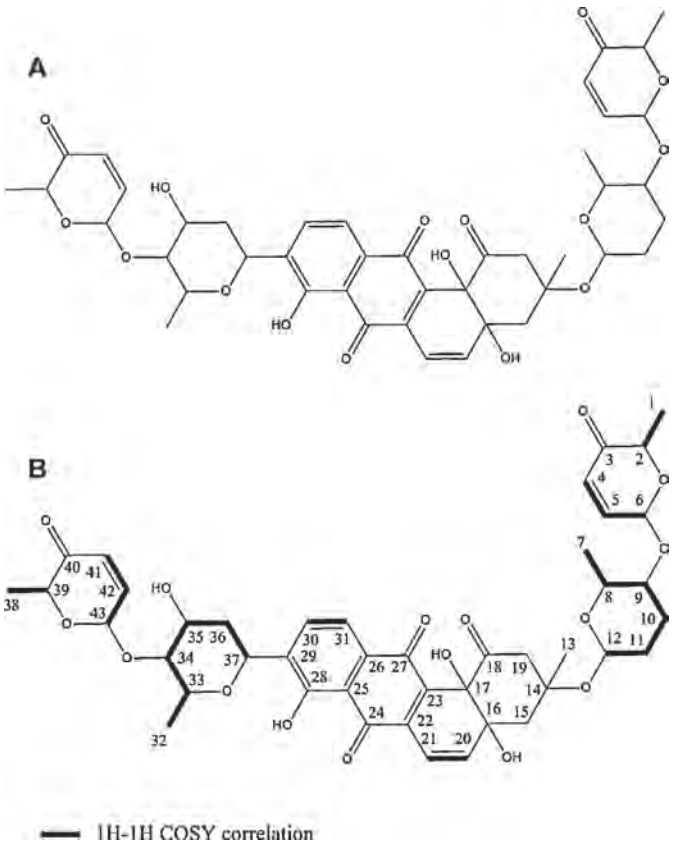

Figure 3 Structure of antibiotic P41B (A) and COSY correlations (B).
Structure de l'antibiotique P41B (A) et corrélations COSY (B).

secreted by the species of this genus may be antibacterial and/or antifungal (aminoglycosides, aromatics, glycopeptides, macrolides, polyenes, etc.) or, have cytostatic and antitumor properties (anthracyclines, such as adriamycin and daunomycin for example), and many of them have found a fairly significant therapeutic application [10]. The same species of actinomycete can produce several antibiotics (sometimes belonging to different classes); however, the same antibiotic can be produced by very different species and sometimes even different genera [11,22]. Therefore, this shows that the correlation between the production of bioactive molecules and taxonomy is weak.

Table 2 Minimum inhibitory concentrations (MICs) of the antimicrobial compounds produced by the strain PAL114 against several target microorganisms.
CMI des composés antimicrobiens produits par la souche PAL114 contre plusieurs microorganismes-cibles.

Target microorganisms	MIC (μg/mL)[a]	
	P41A	P41B
Saccharomyces cerevisiae ATCC 4226	30	30
Candida albicans M1	50	50
C. albicans M2	50	50
C. albicans M3	30	30
C. albicans IPA200	50	50
Aspergillus carbonarius M333	75	75
A. flavus AF3	100	100
Fusarium culmorum FC200	75	75
Penicillium glabrum PG1	75	75
Bacillus subtilis ATCC 6633	30	30
Staphylococcus aureus S1	50	50
Klebsiella pneumoniae E40	> 100	> 100

[a] MIC values represent the average of two repetitions.

The structure of natural antifungal compounds produced by *Streptomyces* (and other microbial genera) can be polyenic or non-polyenic. However, the number of these molecules used in therapy is quite limited because of their toxicity and sometimes their instability and insolubility [4], hence, there is a need to continue the search for interesting new antifungal molecules.

The strain PAL114 produces two molecules with antifungal and antibacterial activities. The structure of these two molecules, named P41A and P41B, was determined by NMR and mass spectrometry, and they appear to be saquayamycin A and C, respectively. They are antibiotics belonging to the family of anthracycline, class of angucycline and group of aquayamycin [15]. Among aquayamycin-group antibiotics, saquayamycins are the most closely related to vineomycin A1 [24]. They are known to be produced by actinomycetes, especially *Streptomyces nodosus*, which produces saquayamycins A, B, C and D [24]. It has been reported that saquayamycin Z was produced by some strains of the genus *Micromonospora* [2].

Saquayamycins are known as inhibitors of farnesyl-protein transferase, an enzyme involved in cell division [14].

Therefore, they are cytotoxic, and used as anticancer agents in chemotherapy [1,14]. Squayamycins A, B, C and D are active against adriamycin-sensitive and adriamycin-resistant sublines of P388 leukemia cells, and it has been reported that saquayamycin A is particularly very active against cancerous prostate cells [20,24]. Adriamycin is an anticancer compound developed in the 1960s, belonging to the anthracycline family, produced by actinobacteria of the genus *Streptomyces* [27]. All saquayamycins are also active against Gram-positive bacteria [2,24], but no antifungal activity was reported in the literature. This is the first time that the antifungal activity (especially against yeasts) of saquayamycins is highlighted.

The results obtained in this work are interesting because of the resistance of strains of *C. albicans* (M1, M2, M3 and IPA200) used in our experimentation to cycloheximide, itraconazole, nystatin, terbinafine and thioconazole.

However, it should be noted that although the antifungal activity of compounds P41A and P41B is interesting in vitro, it is necessary to continue in vivo studies to really evaluate their effectiveness, and it is only from these results that the prospects for clinical application could be considered.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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